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An improved kidney dissociation and reaggregation culture system results in nephrons arranged organotypically around a single collecting duct system

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Methods for constructing engineered "tissues" from simple suspensions of cells are valuable for investigations into basic developmental biology and for tissue engineering. We recently published a method for producing embryonic renal tissues from suspensions of embryonic mouse renal cells. This method reproduced the anatomies and differentiation states of nephrons and stroma very well; it had the limitation, however, that what would, in normal development, be a single, highly branched collecting duct tree leading to a ureter developed, in the engineered system, as a multitude of very small collecting duct trees. These were isolated from each other and therefore would not be effective for draining urine to a common exit, were the tissue to be supplied with blood and physiologically active. Here, we report an improvement on the original method; it results in the formation of nephrons arranged around one single collecting duct tree as would happen in a normal kidney.

Introduction

Developing methods for reconstructing organ rudiments from simple suspensions of cells is important for several reasons. For basic science, it allows researchers to investigate processes of self-organization;[1,2] it also allows them to make fine-grained chimaeras of wild-type and mutant cells to determine whether mutations act in a cell-autonomous manner and whether they bias a cell's choice of fates. For clinical application, an ability to reconstruct organ rudiments from suspensions of cells that have no a priori spatial information is critical to the aim of producing tissues de novo from stem cells cultured in bulk.[3,4]

Recently, we published a method for reconstruction of embryonic kidney tissues from suspensions of cells.[5] For this method, cell suspensions are obtained by enzyme-assisted disaggregation of E11.5 mouse kidney rudiments, followed by temporary pharmacological inhibition of ROCK to reduce loss of cells during this single-cell suspension phase of the experiment. The suspended cells are reaggregated, and they form tubes that express markers typical of ureteric bud/collecting duct. Near these tubes, nephron progenitors form from the mesenchyme and go through their normal morphological sequence of development to produce nephrons with defined Bowman's capsules, proximal tubules and distal tubules; each expresses specific markers in their usual stages and places. The nephrons connect to the nearby ureteric bud/collecting duct structures to make a continuous lumen, as in normal development. In our original report and also in a second paper,[6] we demonstrated the utility of the system for making fine-grained chimaeras and for siRNA-mediated knockdown of gene expression.

The system, as we described it, has one serious anatomical deficiency: because the ureteric bud-derived cells reaggregate to form multiple small branching ureteric buds rather than one large one, the normal tree-like arrangement of the kidney is missing. At high magnification, a section of the reaggregated tissue is difficult to distinguish from a section of the cortex of a normal developing kidney, but at low magnification, the difference is obvious; there is no one, dominating collecting duct tree and, therefore, none of the cortical-medullary differences in tissue organization that would normally be imposed by such a tree. Functionally, this deficiency would be serious for any attempts towards practical renal tissue engineering, because urine made by the nephrons needs to drain away via the collecting ducts; multitudes of isolated small collecting ducts will not achieve this, whereas a single collecting duct system leading to a ureter would. The cortico-medullary organization imposed by a single collecting duct tree on the kidney is also important in a normal kidney for water recovery by countercurrent multiplication.

Work published by Grobstein over half a century ago suggested that it is possible to combine intact metanephrogenic mesenchyme tissue with an intact ureteric bud and induce development of both nephrons and ureteric bud.[7] Similarly, Auerbach

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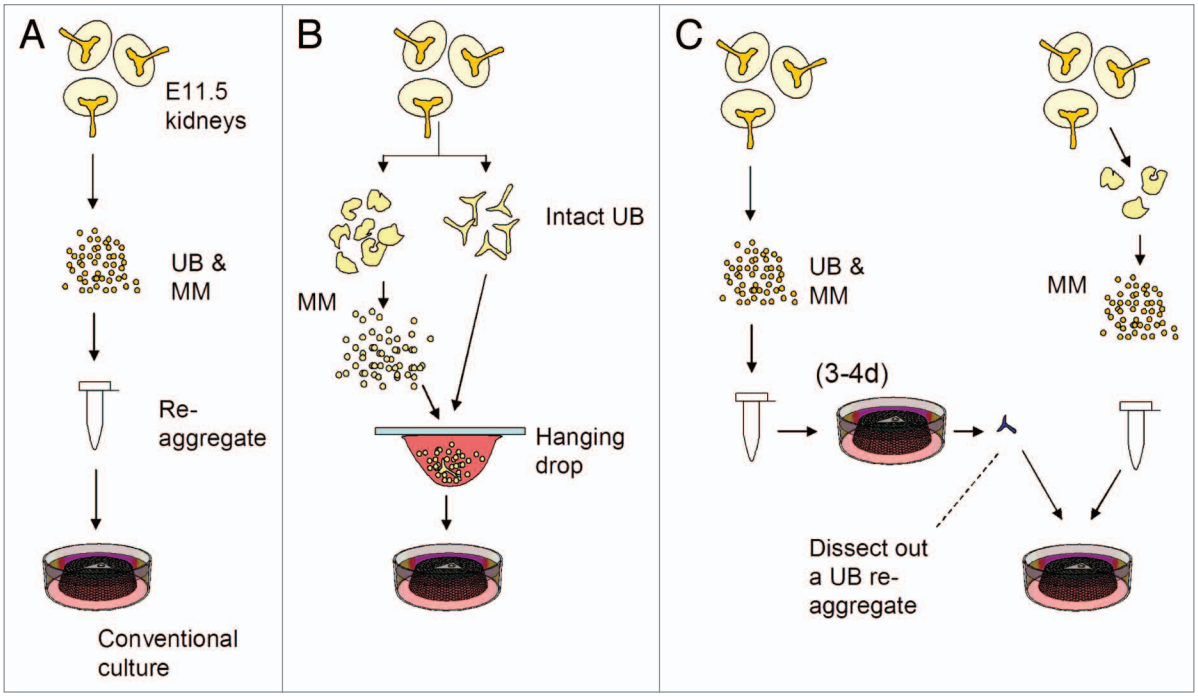

Figure 1. Diagrams to represent the original disaggregation–reaggregation method and the improved methods reported here. (A) Depicts the original method of Unbekandt and Davies,[5] in which whole E11.5 kidneys are disaggregated, reaggregated and cultured. (B) Depicts "method 1" of this paper, in which disaggregated mesenchyme cells (MM) are combined with intact ureteric buds (UB) in hanging drops then cultured. (C) Depicts "method 2" of this paper, in which the original method is used first to create reaggregated tissue, then a single "ureteric bud" is dissected from the reaggregate and combined with reaggregated mesenchyme.

and Grobstein showed that it was possible to combine disaggregated metanephrogenic mesenchyme with a surrogate inducer (spinal cord rather than ureteric bud) and still obtain nephrons.[8] Here, we combine these classical approaches with our modern, pharmacology-assisted dissociation-reaggregation method[5] to generate reaggregate tissues based on a single ureteric bud. We do this two ways (**Fig. 1**); in one, disaggregated mesenchyme is recombined with an intact ureteric bud, and in the other, disaggregated mesenchyme is combined with a single ureteric bud fragment that has reaggregated from a previous round of disaggregation and reaggregation. Both methods result in the reconstruction of organ rudiments that are based on a single branched collecting duct tree; the first method is easier, while the second is more powerful, in that it allows formation of the entire renal structure from simple suspensions of cells.

Results

Method 1: combining intact ureteric bud with dissociated metanephric mesenchyme results in an organotypic arrangement of tissues. Our first method combines an intact ureteric bud, isolated straight from an embryo, with a disaggregated and reaggregated mesenchymal compartment. Metanephric mesenchyme was isolated from E11.5 mouse kidneys and was reduced to a suspension of individual cells. The suspension was then placed in a hanging drop to which a ureteric bud, also isolated from E11.5 kidneys but not disaggregated, was added.[5] Cells placed in the hanging drop congregated at its bottom to make a coherent mass (**Fig. 2A**). After a day, this mass was transferred to a normal renal organ culture environment, consisting of a polycarbonate filter at the surface of medium.

Under these conditions, mesenchyme of the cultures formed a number of developing nephrons located near to the upper branches of the single collecting duct tree (**Fig. 2B**). This arrangement contrasts with the random disposition of nephrons and small "ureteric buds" that is produced when an entire kidney rudiment is disaggregated and reaggregated (**Fig. 2C**).[5] The general layout of the tissues compares well with those of embryonic kidneys cultured intact using the traditional method of growing them on filters at the surface of medium[10,11] (**Fig. 2D**). There is, though, some difference in the shape of the collecting duct system: some of it initially extends around the edge of the aggregate, and some branches, therefore, extend inwards.

Method 2: combining a single reaggregated ureteric bud cyst with reaggregated mesenchyme results in an organotypic arrangement of tissues. Our second method uses only tissues that have been reaggregated from cell suspensions. The first step is a conventional disaggregation and reaggregation of a complete kidney, using the technique already described in reference 5. As reported before, after 4 or 5 days (1 in ROCK inhibitor and 3 or 4 without), many ureteric bud cysts and nephrons form in these reaggregates (**Fig. 3A and C**). Ureteric bud cysts were then isolated, by microdissection, from these reaggregates and were placed on a filter at the surface of conventional medium. To each ureteric bud was added a pellet of mesenchyme, reaggregated from a suspension of individual, disaggregated mesenchyme cells.

Under these conditions, the reaggregated ureteric bud "cyst" developed into a single, extensively branched ureteric bud/collecting duct system over 3–4 days (**Fig. 3B and D**). Adjacent to the branches of the bud, nephrons formed, showing their normal morphology, and they appeared to connect to the bud/collecting duct system (**Fig. 3B and D**). The branching was directed outwards as usual. Essentially, the anatomy is typical of that of a normal, intact embryonic kidney (**Fig. 2D**).

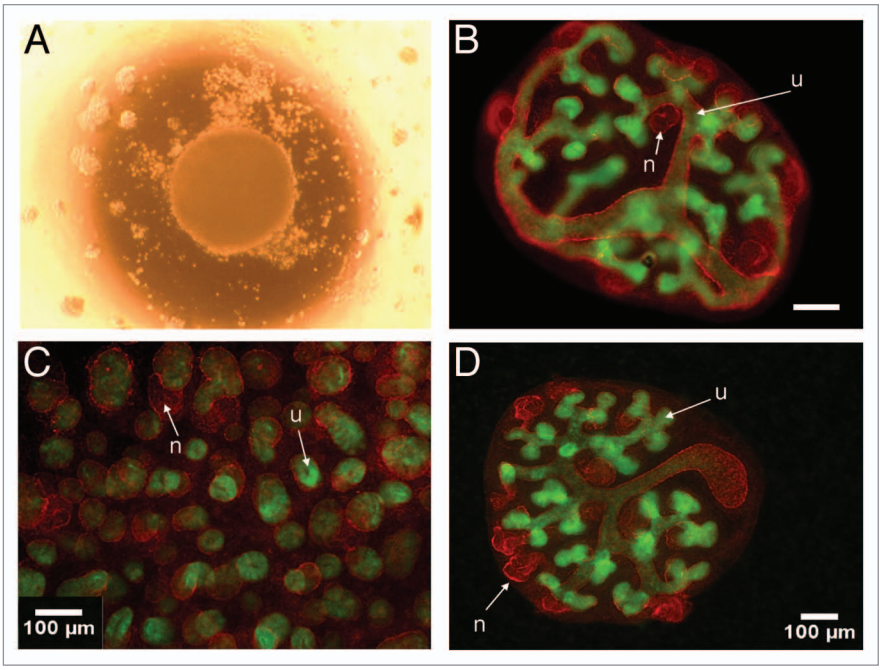

Figure 2. Method 1: combination of reaggregated mesenchyme with an intact ureteric bud. (A) Metanephrogenic mesenchyme cells form a compact mass at the bottom of a hanging drop. (B) When a ureteric bud is added to the hanging drop, and the resulting tissue mass is transferred to a conventional organ culture system 24 h later, it goes on to develop over the next 3 days to produce a branching ureteric bud ("u" expressing calbindin-D^{28k}, green as well as laminin, red) and comma- and S-shaped developing nephrons (n, red only) form near the tips of some of its branches. (C) In a standard reaggregate, made by disaggregation of the complete kidney,[5] short tubules of both types are present (u, n as before), but large-scale organization is not apparent. (D) An intact kidney in organ culture, shown for the purposes of comparison; labels as before.

Discussion

In this short report, we have demonstrated two methods for producing reaggregated embryonic renal tissues that have a significantly more organotypic arrangement of tissues than do tissues produced in the basic dissociation and reaggregation method.[5,6] The key improvement is that these techniques result in nephrons being arranged as they should be in relation to a single, branching ureteric bud.

The first method, use of an intact ureteric bud, is the simplest and quickest, and it is suitable where there is no reason for dissociating the bud in the first place. Experiments on the basic developmental biology of nephrons, or on the ability of putative stem cells to integrate into nephrons and produce their specialized cell types would be examples of such experiments. By including a step that involves the mesenchyme being a suspension of individual cells, the method allows the mixing of cells with different genotypes to make chimaeras for testing the cell autonomy of mutations or the effects of mutation in fate choice, but only within the mesenchyme-derived compartment (we have already demonstrated use of these techniques for the whole kidney reaggregation system[5,6]).

The second method is more involved, but it retains the full power of the original dissociation–reaggregation method to produce all of the tissues from simple suspensions of cells. This will allow the production of fine-grained chimaeras of all tissue types, even using different genotypes for mesenchyme and bud. Importantly, it means that a kidney that is properly arranged around a single ureteric bud/collecting duct tree can be made from simple suspensions of cells, something that might be very important for building renal tissue from cultures of stem cells, which is a major research goal in the field.[12-15]

The basic disaggregation–reaggregation method facilitates research on the processes of self-organization that take place on small scales, for example, in the formation of nephrons. The systems described here, which have correctly arranged, large-scale anatomies, could extend this to accommodate research into processes of self-organization at the whole-organ scale, for example cortical-medullary organization, directional growth of loops of Henle, etc., in culture.[16] We therefore believe them to be of potential importance to basic research as well as tissue engineering. Furthermore, being culture-based, they have the potential to contribute to a reduction in in vivo experimentation, at least in the initial stages of exploratory research.

Materials and Methods

Culture medium. Kidney culture medium (KCM) consisted of Eagle's MEM (Sigma, cat #M5650) with 1% Penicillin/Streptomycin (Sigma, cat# P4333) and 10% foetal calf serum (BioSera).

Dissection and disaggregation of embryonic kidney rudiments. E11.5 embryonic kidney rudiments were dissected, dissociated and then reaggregated, as we have described before.[5] For experiments that required them, intact ureteric buds were isolated by incubating kidneys in 2x Trypsin/EDTA solution (Sigma, cat# T4174) in Eagle's MEM (Sigma, cat# M5650) for 2 min at 37°C, transferring them to KCM to quench the trypsinization and pulling ureteric buds away from their mesenchymes using 25-gauge needles. Ureteric buds were examined carefully to ensure that they were clean of mesenchyme cells (most were, as the trypsinization separates the two tissues along the basement membrane). Mesenchymes destined for diasaggregation

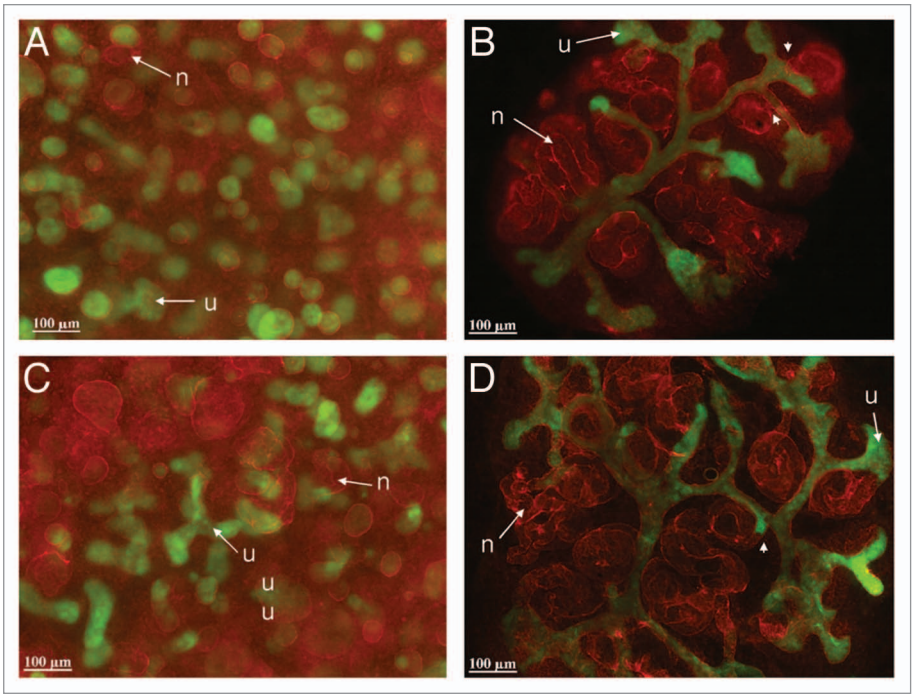

Figure 3. Method 2: both ureteric bud and mesenchyme coming from cell suspensions. To prepare reaggregated ureteric buds for later combination with mesenchyme, dissociated E11 mouse embryonic kidney cells were reaggregated and cultured for one day with 1.25 µM glycyl-H1152-dihydrochloride and then for (A) 3 or (C) 4 days in plain kidney culture medium. Many cyst-like ureteric bud structures (u, green, with red border) and primitive nephrons (n, red border only) form in these conditions: the green immunostain detects CalbindinD^{28K,} and the red detects laminin. A single ureteric bud structure was isolated from these reaggregates and cultured for (D) 3 days or (B) 4 days with a reaggregate of dissociated mesenchymal cells. Under these conditions, the ureteric bud "u" structure grows and branches to make a tree characteristic of normal developing kidneys, and many nephrons "n" can be observed connecting to them (arrowheads).

were isolated from E11.5 kidney rudiments. Ten to fifteen mesenchymes were incubated for 2 minutes at 37°C degrees in PBS with 0.5x Trysin/EDTA and then in KCM to quench the trypsin effect. They were then placed in a 0.5 ml microcentrifuge tube containing 200 µL KCM and dissociated by vigorous trituration using a 200 µl Gilson tip, until a homogeneous suspension with no visible clumps was obtained. The cell suspension was filtered through a 40 micron cell strainer (Millipore), and the total number of cells was counted. Batches of about 10^5 mesenchymal cells were reaggregated by centrifuging them 3,000 rpm (800 g) for 2 minutes. For recombination with an intact ureteric bud (i.e., "method 1"), the resulting pellet was resuspended in 25 µl KCM, which is enough for two hanging drops; for some hanging drop experiments, 10 µmol/L of the ROCK inhibitor Y27632 (Sigma) was included, but this was later found this to be unnecessary for use with hanging drops that included intact ureteric buds. For recombination with a ureteric bud reaggregated cyst (i.e., "method 2"), the pellet was detached from the border of the tube, care being taken to keep it intact.

Reaggregation of mesenchyme cell suspensions with an intact ureteric bud. Initial recombination of ureteric buds and mesenchyme suspensions was performed by a variation of the hanging drop method used by Sainio et al. for culture of ureteric buds.[9] Twenty-five µL of mesenchyme suspension, made as above, was pipetted onto the inside of a 3.5 cm Petri dish lid to make two similarly sized, separate drops and a single intact ureteric bud was added to each. The lid was inverted and placed over the Petri dish containing 2 mL of KCM to buffer the hanging drops against drying out. The dish lid was tapped gently to encourage suspended cells to sediment to the bottom of the hanging drop, and the drops were incubated overnight at 37°C, 5% CO_2. The recombined tissues were then transferred carefully, by pipette, to the top of a filter on a Trowell screen (as described for reaggregated pellets[5]) and incubated for 3–5 days in plain KCM (with no Y27632).

Reaggregation of mesenchyme reaggregates with ureteric bud reaggregates. First, conventional whole-kidney reaggregates were produced using the standard method.[5] These were cultured for one day in the presence of ROCK inhibitor (1.25 µM glycyl-H1152-dihydrochloride) and then for 3 or 4 days in KCM. Single ureteric bud cysts were identified by their size and shape and were dissected manually from these reaggregates. Each individual cyst was placed in culture on a polycarbonate filter at the culture medium/atmosphere interface and covered with a pellet containing about 10^5 reaggregated mesenchymal cells, prepared as described above. The recombined tissues were incubated for 3 or 4 days in plain KCM at 37°C, 5% CO_2. A culture time of 3 days was applied when the initial conventional reaggregate, from which the ureteric bud rudiment was taken, was 1 day with ROCK inhibitor and 4 days without, and a culture time of 4 days was applied when the initial culture was 1 day with ROCK inhibitor and 3 days without, so that the total culture time from beginning to end from the point of view of the ureteric bud was always 8 days.

Immunohistochemistry. Tissues were fixed in methanol at -20°C and either stored in this liquid at this temperature until needed or left for at least 10 minutes for fixation. Fixed specimens were washed in PBS for 30 minutes at room temperature and then incubated with a solution of primary antibodies diluted 1 in 100 in PBS overnight at 4°C. Primary antibodies were mouse anti-Calbindin (ab82812, Abcam) and rabbit anti-Laminin (L9393, Sigma). Samples were washed for 30 minutes in PBS and then incubated overnight at 4°C with secondary antibodies diluted 1 in 100 in PBS. Secondary antibodies were goat anti-mouse IgG-FITC (F0257, Sigma) and goat anti-rabbit IgG-TRITC (T5268, Sigma). A final wash in PBS was performed for at least 30 minutes. Images were obtained with Zeiss Imager A1 (Carl Zeiss, Welwyn Garden City, UK), Leica Ortholux II (Leica Microsystems GmbH,

Wetzlar, Germany) and HUND Wetzlar Wilovert (Helmut Hund GmbH, Wetzlar, Germany) microscopes. Whenever necessary, separate channels were merged, aligned and modified using the NIH software ImageJ version 1.43.

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Note

Because this manuscript includes this journal's Editor-in-Chief amongst its authors, an anonymous peer review of this paper was organized, and all editorial decisions were made, by another member of the Editorial Board.

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